Impact of Malaysian Continental Drift on the Genetic Diversity of Horseshoe Crab Inferred through mtDNA Sequence Analysis

M. Rozihan (Corresponding author)
Department of Aquaculture, Faculty of Agriculture
Universiti Putra Malaysia, UPM 43400, Serdang, Selangor, Malaysia
E-mail: rzihan@hotmail.com

E. Ismail
School of Bioscience and Biotechnology
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
UKM Bangi 43600, Selangor D.E. Malaysia
E-mail: bajphd@yahoo.co.in

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Abstract

An attempt was made to examine the influence of Malaysian continental drift on the genetic and haplotype diversity of Malaysian horseshoe crab (*Tachypleus gigas* [Muller, 1785]) distributed along the east coast of Peninsular Malaysia and distant Borneo Island. Mitochondrial DNA (AT rich region = 369bp) analysis showed that *T. gigas* has higher haplotype diversity in peninsular Malaysia compared to east Malaysian (Sarawak) samples. Highest haplotype diversity ($h \pm SD$) was observed among the Terengganu samples (0.813 ± 0.071) followed by Pahang samples (0.813 ± 0.065). There were no difference in nucleotide diversity (π) noted in east cost of Peninsular Malaysian (PM) samples. Overall haplotype ($h \pm SD$) and nucleotide ($\pi \pm SD$) diversity of *T. gigas* in PM samples including Sarawak was 0.827 ± 0.051 and 0.0078 ± 0.0014 respectively. A total of 6 unique haplotypes were recorded of which 2, 2 and 1 were unique to Terengganu, Sarawak and Pahang samples respectively. Pair wise haplotype frequency (F_{ST}) value was not statistically significant ($P > 0.05$) for all the groups indicating the limited gene flow among the population. In addition phylogenetic scrutiny visibly clustered *T. gigas* samples from *T. tridentatus* samples representing sound phylogenetic signals in mtDNA AT rich region. The findings from this study have important implications for proper management and conservation of horseshoe crab in Malaysia.

Keywords: Haplotype diversity, Nucleotide diversity, mtDNA AT rich region, *Tachypleus gigas,* Genetic conservation

1. Introduction

Horseshoe crabs are a unique group of animals remarkably retaining their genetic makeup virtually unaffected over millions of years (Kamaruzzaman et al., 2011; John et al., 2010a). They are marine chelicerate arthropod belong to the class merostomata. Despite their name, they are more closely related to spiders, ticks and scorpions than to crabs (kamaruzzaman et al., 2011). There are four extant species of horseshoe crabs, *Tachypleus tridentatus, Tachypleus gigas*, *Carcinoscorpius rotundicauda* and *Limulus polyphemus.* Demographic data showed their global distributory pattern where Atlantic horseshoe crab (*L. polyphemus*) most commonly found in Gulf of Mexico, Southeast Asian horseshoe crab (*T. gigas)* found in the shores of the bay of Bengal particularly along the coast of Orissa (India) to Indo-China, North Vietnam, Borneo and Celepes), *T. tridentatus* (Northern shores of Japan up to South Vietnam and along the Western islands of the Philippines) and (Mangrove horseshoe crab) *C. rotundicauda* (Northern shores of the bay of Bengal to the Southern coast of the Philippines) where they inhabit in the continental shelf region within 47.7km upto 312km (Elizabeth, 2001; Chatterji et al., 1992). Out of

four extant species of horseshoe crabs, 3 are inhabiting Malaysian coastal waters while the distribution of *T. tridentatus* is restricted to East Malaysian coasts (Sabah and Sarawak regions) (John et al., 2010).

In recent decades, various molecular tools have been widely used in variety of generic diversity studies, population structure prediction including their phylogeny and phylogeographic analysis (Rozihan and Ismail, 2011). Nowadays, phylogeography has become a powerful field of research revealing the principles and processes determining the geographic distribution of genealogical lineages, within and among closely related species (Avise, 2000). The main goal of this 'comparative phylogeography' has been to search for concordant geographical distribution among lineages within different species, which would indicate the influence of common historical factors. Recent comparative phylogeographic studies have found that vicariance phenomena has major role in structuring species assemblages and their genetic diversity in geographically distinct population of same species (Arbogast and Kenagy, 2001). It has been widely argued that the restricted population of *T. tridentatus* in East Malaysia might probably due to the continental drift that took place over 1000 years back (Jeannette and Ridder-Numan, 1996; Whitten et al., 1996). Recently Ward et al., (2005), suggested that molecular genetic analysis is a powerful tool for investigating genetic differentiation within and among the population of species which would eventually give genetic history of the population under study. Many studies were conducted to evaluate the genetic break between the northern and southern populations of American horseshoe crabs (*L.polyphemus*) along the Florida state by using allozyme (Giribet et al., 2001), mitochondrial DNA restriction fragment length polymorphism (RFLP) analysis (Pierce et al., 2000), and microsatellite (King and Eackles, 2004). However, related studies on their conspecific in Malaysian coast are still scanty except in West coast (Rozihan and Ismail, 2011).

The mtDNA AT rich region is a highly variable, non coding region that is useful for phylogeographical studies and population genetic surveys, although the high AT content poses technical and analytical problems (Vila and Bjorklund, 2004). Study had been carried out to predict the population structure of tri spine horseshoe crab (*T. tridentatus*) using mtDNA AT rich region as a marker gene in Taiwan (Yang et al., 2007) and on *T. gigas* along the west coast of Malaysia (Rozihan and Ismail, 2011). Present work is the first attempt to evaluate the genetic diversity of *T. gigas* distributed along the East coast of peninsular Malaysia together with east Malaysian sample.

2. Materials and Methods

2.1 Study site and sample collection

A total of 3 sampling sites were located (2 sites from peninsular Malaysia and 1 from east Malaysia [Sarawak]) and 30 *Tachypleus gigas* samples were collected from the nesting beaches (Terengganu = 14; Pahang = 10 and Sarawak = 6) during 2008 (Figure 1 & Table 1). Samples were identified, weighed, sexed and morphometric, meristic characteristics were measured. A piece of walking leg was excised from each sample using sterilized scissors and the internal soft tissue was preserved in 95% ethanol (John et al., 2010). All the animals were released back into the ocean in live condition to make sure their sustainable fishery.

2.2 Wet lab work

DNA isolation was performed by following the published method (John et al., 2010). The complete AT rich region of mtDNA was amplified by a pair of primers, Hb-trna (5'-GAGCCCAATAGCTTAAATTAGCTTA-3') and Hb-12S (5'- GTCTAACCGCGGTAGCTGGCAC-3') (Yang et al., 2007). Amplification reaction was conducted in 50 µl buffer supplied with the enzyme and under the conditions recommended by the manufacturer (Invitrogen, Germany). Each 50 µl volume contained 50 mM KCl, 10 mM Tris (pH 9), 3 mM $MgCl_2$ 0.2 mM each dNTP, 0.04 mM each primer, 0.033 units of Tag polymerase, 1 µl DMSO and 50 ng of mtDNA. The thermocyclic conditions for PCR included the initial denaturation at 94ºC for 1 min, five cycles of 94ºC for 30 sec, annealing at 45ºC for 40 sec, and extension at 72ºC for 1 min, with a final extension at 72ºC for 10 min, followed by indefinite hold at 4ºC. Following PCR, about 10µl of PCR product with 2µl of bromo thymol blue were added to 2% agarose gel, prepared with 2.5 µl of 1% Ethidium Bromide and electrophorized at 90V until the dye moved for 6cm in the gel. The gel was moved to gel doc system for viewing the amplicons with the aid of UV trans-illuminator. Final PCR product was sequenced using ABI 3730xl sequencer and obtained chromatogram was edited via ABI sequence scanner software 1.0v.

2.3 Data analysis and software prediction

All data analyses were analyzed using Arlequin 3.0v for a Macintosh platform (Excoffier et al., 2005). Unique haplotypes and all transitions and transversions were counted. Haplotype diversity (*h*), nucleotide diversity (Shimatani, 1999), and their standard errors were calculated. Pairwise F- statistics (F_{ST}) were calculated as genetic distances based on pair wise differences between populations using Dna SP software 4.50.3v (Rozas et al., 2003).

An indirect estimate of gene flow was calculated based on the equation $N_em = 0.5 \times [(1/F_{ST}) - 1]$ where N_e is the effective number of females and m is the migration rate. Percentage of AT was calculated using Bio edit software (Hall, 1999) and Transition:Transversion (Ti:Tv) ratio was calculated using MEGA 4.0 (Tamura et al., 2007).

3. Results and Discussion

We identified 14 haplotypes among the *T.gigas* samples from East coast of peninsular Malaysia (PM) including Sarawak. Among these haplotypes 2 (TG2, 4), 2 (TG8, 9) and 1 (TG3) were unique to Terengganu, Sarawak and Pahang samples respectively (Table 2). The complete data matrix comprised of these 14 haplotypes as well as with *T.tridentatus* sample as an out group clearly clustered *T.tridentatus* in separate branch (Figure 2) indicating high phylogenetic cues in mtDNA AT rich strand. The observed mean transition to transversion ratio in the mtDNA AT rich strand was 2.6. The observed mean AT content in the controlled region (mtDNA AT rich region) was 86.03% (Table 3). Haplotype diversity was comparatively higher in PM samples than east Malaysian *T. gigas*. Greater haplotype diversity was noted in Terengganu horseshoe crab populations (0.867 ± 0.071) followed by Pahang samples (0.813 ± 0.065) and Sarawak samples (0.8 ± 0.172). There were no changes in the nucleotide diversity observed between the PM samples. These two observation clearly indicated the restricted gene flow together with higher polymorphic cites in the mitochondrial genome within the PM crab population in contrast to PM and East Malaysian (EM) samples. Similar observation was noted in horseshoe crab population from Malaysian west coast samples where the geographically closer population had restricted gene flow (Rozihan and Ismail, 2011; Smith et al., 2009). Yang et al., (2007) has observed that *T. tridentatus* population from closer geographical area of Taiwan coastal waters showed almost similar level of nucleotide diversity which eventually led to the restricted gene flow among the population. The lowest nucleotide diversity in Sarawak crab samples might probably due to 1. Their recent colonization in east Malaysia, 2. The continental drift that separated the PM and EM might have carried along a patchy population of *T. gigas* towards the EM together with the complete shifting of *T. tridentatus* population from East coast of PM. Similar results were observed in previous study (Hewitt, 1999). Avise, (2000) also proposed that a rapid expansion from refugial populations involves serial bottlenecks with progressive loss of allelic diversity resulting in less genetic diversity among populations living in the more recently colonized places.

3.1 Gene flow and migratory rate

The fixation index (F_{ST} value) between Pahang vs Terengganu samples were lower (0.038) indicating restricted gene flow between these populations. This observation was also proved by migratory rate per generation between populations (N_em) which revealed the higher migratory rate between Pahang vs Terengganu samples (12.658) (Table 4). This analysis clearly proved the greater migration of horseshoe crab samples along the east coast of peninsular Malaysia while the migration between PM to EM is highly restricted. Similar observations were also recorded in other aquatic organisms (Wong et al., 2011; Van der Kuyl et al., 2005).

4. Conclusion

Present study proved the restricted geographical gene flow among the *T. gigas* population along the east coast of peninsular Malaysia. The influence of Malaysian continental drift which separated peninsular Malaysia from East Malaysia (Borneo) had apparent effect on the dispersal of *T. gigas* towards Sarawak region. However, additional molecular data together with increased sample size would pave a way in exploring the dispersal pattern of horseshoe crab along the Malaysian coast line. This genetic information of the local populations could be used to enact different conservation strategies for their sustainable fishery along the Malaysian coast line.

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Table 1. Detailed information of the sampling location and geographical information (*Note:* EPM: East Peninsular Malaysia, EM: East Malaysia [Sarawak])

Geographical region	Sampling site	Sample ID	Site locations (GPS reading)
EPM	Pahang	PAH	N 03^0 31.988' E 103^0 27.534'
EPM	Terengganu	TER	N 05^0 41.000' E 102^0 42.594'
EM	Sarawak	SAR	N 01^0 38.944' E 110^0 28.508'

Table 2. Variable sites found in a fragment of AT-rich region of *Tachypleus gigas* and their distribution in the population.

Haplotypes	Nucleotide positions																Populations			Total
	66	76	96	101	122	131	138	151	154	182	194	196	209	286	299	308	PAH	TER	SAR	
TG1	G	G	T	A	A	T	C	C	A	T	A	A	G	A	T	C	5	2	1	8
TG2	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	T		1		1
TG3	*	A	C	*	*	*	*	*	*	*	G	*	*	G	*	*	1			1
TG4	A	*	*	G	*	*	*	*	*	*	*	*	*	*	*	T		2		2
TG5	*	A	*	*	G	*	*	*	*	*	*	G	*	*	*	*	2	2		4
TG6	*	A	*	*	G	*	*	*	*	*	G	*	*	*	*	*	3		3	6
TG7	*	*	*	G	*	*	*	*	*	*	*	*	*	*	C	T	3	3		6
TG8	*	A	*	*	*	*	*	*	*	*	G	*	*	G	*	*			1	1
TG9	*	*	*	G	*	*	*	*	*	*	G	*	*	*	*	T			1	1
Total																	14	10	6	
Nucleotide diversity (Pi)																	0.008	0.008	0.007	
Number of haplotypes (h)																	5	5	4	
Haplotype diversity (Hd)																	0.813	0.867	0.800	
Number of polymorphic sites (s)																	9	7	6	

(*Note*: Sampling stations IDs, PAH = Pahang; TER = Terengganu; SAR = Sarawak; TG1- TG12 represents the observed haplotypes in *Tachypleus gigas*; '*' represents the polymorphic mismatches in mentioned nucleotide position).

Table 3. Localities and molecular characters in *T. gigas* mtDNA AT-rich region

Sampling sites	Number of samples	(A+T)%	Ti:Tv	$h \pm$ SD	$\pi \pm$ SD
Pahang	14	86.0	3:0	0.813 ± 0.065	0.0084 ± 0.0012
Terengganu	10	86.2	3:0	0.867 ± 0.071	0.0084 ± 0.0012
Sarawak	6	85.9	2:0	0.800 ± 0.172	0.0067± 0.0017
	N= 30	86.03	2.6:0	0.827 ± 0.051	0.0078 ± 0.0014

(*Note*: Nucleotide content (A+T%), number of substitutions (Ti, transition: Tv, Transversion), haplotype diversity (h), and nucleotide diversity (π)).

Table 4. Pair wise F-statistic (F_{ST}) values of genetic differentiation and migrants per generation (N_em) values of gene flow among populations. F_{ST} values are above the diagonal and N_em values are below the diagonal

Populations	PAH	TER	SAR
PAH	-	0.038^{ns}	0.046^{ns}
TER	12.658	-	0.265^{ns}
SAR	10.370	1.387	-

Note: * = $p < 0.05$, ns: not significant.

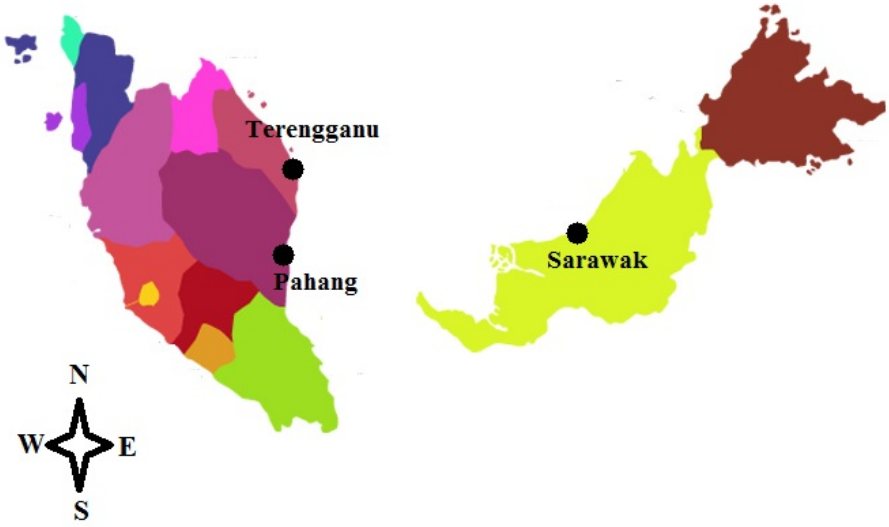

Figure 1. Location of the sampling area

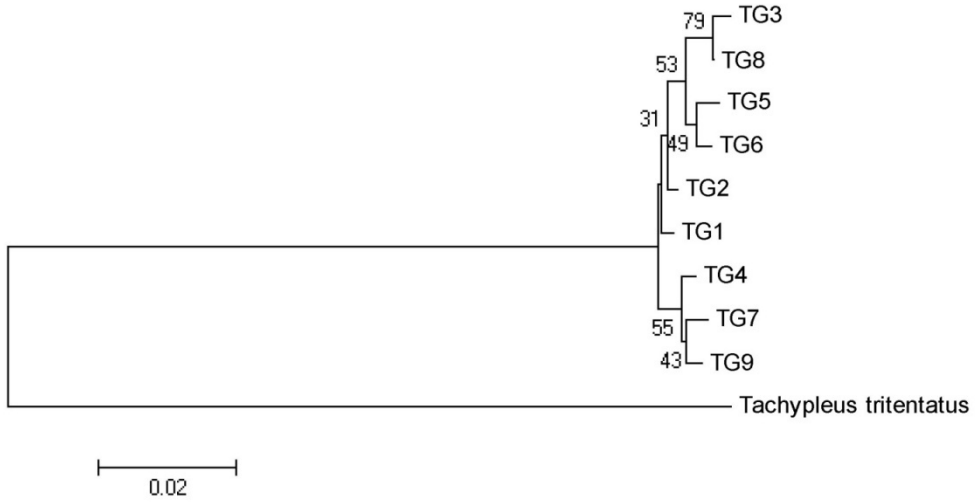

Figure 2. Neighbor-joining (NJ) phylogram showing the relationship among haplotypes of *T. gigas*

The numbers at each node represents the bootstrap percentage values based on 1000 pseudo replications for NJ/MP analyses. *T. tridentatus* used as an out group was clearly clustered in separate branch proves the reliability of the constructed phylogram.